AD_____

Award Number: W81XWH-08-1-0722

TITLE: New Enzyme Prodrug and Methionine-Depletion Combination Therapy of Breast Cancer Designed for Effective Delivery to the Tumor

PRINCIPAL INVESTIGATOR: Roger G. Harrison, Ph.D.

CONTRACTING ORGANIZATION: University of Oklahoma, Norman, OK 73019-0430

REPORT DATE: October 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-10-2010	Annual	15 Sept 2009 – 14 Sept 2010

4. TITLE AND SUBTITLE
New Enzyme Prodrug and Methionine-Depletion Combination Therapy of Breast Cancer Designed for Effective Delivery to the Tumor

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0722

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Roger G. Harrison

rharrison@ou.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Oklahoma

Norman, OK 73019-0430

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The L-methioninase-annexin V fusion protein (FP) was produced from recombinant *E. coli* in good purity and relatively good yield, using the FP gene with a completely correct sequence. As indicated by measuring the dissociation constant (K_d), purified FP binds strongly to human endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells grown *in vitro*. Tests with the enzyme prodrug over a period of 3 days showed significant cell killing at 500 μM SeMet for endothelial cells, 50 μM SeMet for MCF-7 breast cancer cells, and 10 μM SeMet for MDA-MB-231 breast cancer cells; with no FP present, significant cell killing was not observed for the endothelial cells and MDA-MB-231 cancer cells at up to 1000 μM SeMet and for MCF-7 cancer cells at up to 500 μM SeMet. These results provide strong support for the idea that this enzyme/prodrug system will lead to killing of breast tumors. Based on time profiles of the FP in the bloodstream of nude mice and on tests of injecting SeMet i.p at various levels, the FP and SeMet will both be injected at a level of 10 mg/kg for the enzyme prodrug tests in nude mice, which are currently in progress.

15. SUBJECT TERMS
Enzyme prodrug and methionine-depletion therapy for breast cancer, tumor vasculature targeting

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	18	19b. TELEPHONE NUMBER *(include area code)*
U	U	U			

Table of Contents

INTRODUCTION

The focus of this project is on the development of new ways to treat breast cancer with minimal or no side effects. The project aims to develop a novel enzyme prodrug and methionine-depletion combination cancer therapy in which the enzyme L-methioninase is targeted by the human protein annexin V to the breast tumor vasculature, using selenomethionine as the prodrug. Annexin V is known to bind with high affinity to phosphatidylserine (PS) in phospholipids bilayers. PS has recently been shown to be expressed on the external surface of endothelial cells that line the blood vessels in tumors but is not expressed on the external surface of the vascular endothelium in normal organs. The enzyme L-methioninase catalyzes the conversion of methionine to methanethiol, α-ketobutyrate, and ammonia. It also catalyzes the conversion of selenomethionine (SeMet) to toxic methylselenol, α-ketobutyrate, and ammonia. Methylselenol has been shown to be cytotoxic to various cancer cells. To accomplish the specific aims of this project, the L-methioninase-annexin V fusion protein (FP) will be produced and purified, the strength of binding of the FP to human endothelial cells and two breast cancer cell lines *in vitro* will be determined, the cytotoxicity *in vitro* of the FP in combination with the selenomethionine prodrug will be determined for endothelial cells and two breast cancer cell lines, and the FP will be tested in nude mice with tumor xenografts (two breast cancer cell lines) for its effect as an enzyme prodrug by itself and also in combination with methionine-depletion therapy.

BODY

The research accomplishments for the second year of this project are summarized as follows:

Task 1 – Production of the recombinant L-methioninase-annexin V fusion protein to be tested

Two mutations found in the L-methioninase-annexin V gene were corrected using the Stratagene QuikChange XL site-directed mutatagenesis system, as follows: Arg at amino acid number 328 was corrected to Gly, and Cys at amino acid number 416 was corrected to Phe (amino acid number 11 of annexin V). The L-methioninase-annexin V FP with the correct gene sequence was produced by recombinant expression in *E. coli* and purified using the same procedures described in the annual report for year 1 of the project. For a 1 liter batch of cell culture, the yield of purified protein was 22 mg. An SDS-PAGE analysis showing the protein purity at different steps in the purification process is shown in Figure 1. The purified FP (lane 4) was estimated to be >97% using UN-SCAN-IT densitometry software. The recovery of L-methioninase activity from the starting broth was 38%, and the specific activity of the purified protein was 1.6 U/mg protein.

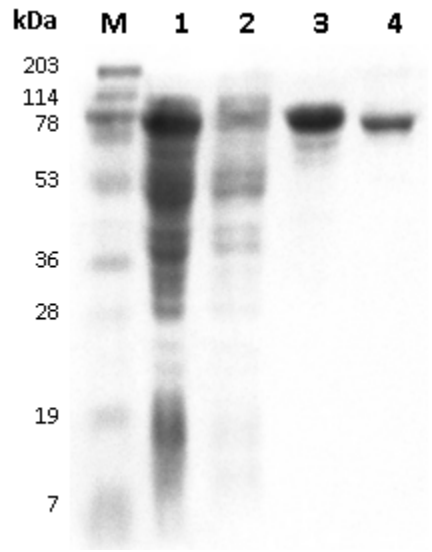

Figure 1. SDS-PAGE gel of methioninase-annexin V purification. An 8% gel with Coomassie blue staining was used to determine approximate molecular mass of 10 µl of purified samples. *Lane 1*, soluble proteins; *lane 2*, first chromatography flow-through; *lane 3*, first chromatography elution; *lane 4*, second chromatography flow-through; *M* marker proteins with molecular masses indicated in kiloDaltons (5 µl).

Task 2 – Test of the function *in vitro* of annexin V in the L-methioninase-annexin V fusion protein

In order to determine whether correcting the two mutations in the FP gene had an effect on the equilibrium binding of annexin V in the FP and on the stability of the FP bound to the three cell lines, these assays were performed using the same procedures as reported in the annual report for year 1. The dissociation constant (K_d) for each cell line tested was obtained from the specific binding data using GraphPad Prism 5 software to give the following results: 0.5 ± 0.2 nM for endothelial cells, 6.2 ± 1.6 nM for MCF-7 breast cancer cells, and 4.9 ± 0.9 nM for MDA-MB-231 breast cancer cells. The equilibrium binding results for the endothelial cells are shown graphically in Figure 2. These results indicate that the binding of the FP to these cells is relatively strong and that the binding to the endothelial cells is stronger than for either of the two breast cancer cell lines.

Data for binding stability of the FP with the mutations corrected is given in Figure 3. This data indicates that the binding of FP declined over 3 days for all three cell lines, with the MDA-MB-231 cancer cells showing the most rapid decline; however, the FP was still present at day 3 for all three cell lines. Cell viability, as measured by the Alamar Blue assay, was found to be linearly proportional to the number of cells (data not shown).

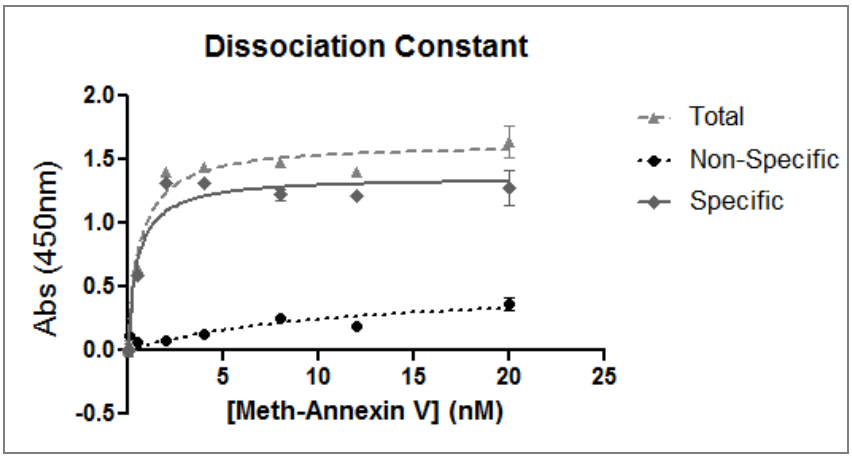

Figure 2: Determination of FP binding strength to exposed PS on HAAE-1 human endothelial cells. FP was biotinylated and streptavidin-HRP was used to quantify the binding. Total binding was obtained by having 2 mM of Ca^{2+} in the binding buffer. Non-specific binding was obtained by removing the Ca^{2+} in the binding buffer and replacing it with 5 mM of EDTA to chelate Ca^{2+}. Specific binding was obtained by subtracting the non-specific binding from the total binding. GraphPad Prism 5 software determined the specific binding to have a K_d = 0.5 ± 0.2 nM. Data are presented as mean ± SE (n = 3).

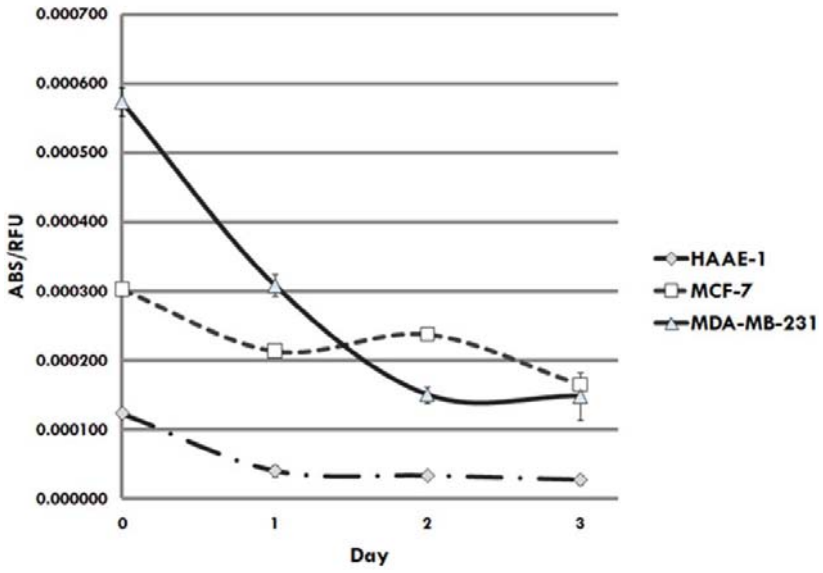

Figure 3. Figure 3: Fusion protein binding stability. The Alamar Blue assay was performed each day, followed by the binding assay to determine the duration of binding of annexin V to the exposed PS on the surface of HAAE-1 human endothelial cells, MCF-7 human breast cancer cells, and MDA-MB-231 human breast cancer cells. FP was found to stay bound to the cell lines for at least 3 days, the period of the experiment. ABS is the absorbance at 450 nm that is measured in the binding assay. RFU is relative fluorescence units, measured by the Alamar Blue assay for cell viability. Data are presented as mean ± SE (n = 3).

Task 3 – Test of the anticancer activity *in vitro* of the L-methioninase-annexin V fusion protein in combination with selenomethionine prodrug on endothelial cells and breast tumor cells

The ability of the enzyme/prodrug system to eliminate human endothelial cells and breast cancer cells was evaluated using the same procedures as reported in the annual report for year 1 for the FP with the two mutations corrected. Concentrations of SeMet ranging from 0 – 1000 μM were studied (Figures 4, 5, and 6). The methionine concentration in the medium was set at a level (1000 μM) that would not lead to a significant decrease in cell viability because of methionine depletion with FP present. Each of the cell lines metabolized the Alamar Blue to produce a fluorescence that was measured to quantify total cell viability. The fluorescence data from the Alamar Blue assay was expressed as a percentage of the fluorescence for the cells with no FP and 0 μM SeMet (control). Cells that were treated with different SeMet concentrations but no FP were compared to the control on the same day, whereas cells that had the FP were compared to cells with the same SeMet concentration but no FP on the same day.

The cytotoxicity results for the endothelial cells, the primary target in the tumor for this enzyme prodrug therapy, are shown in Figure 4. Treatment with the FP gave significant cell killing for 500 and 1000 μM SeMet at days 1, 2, and 3 ($p < 0.001$). With no FP present, significant cell cytotoxicity was not observed at the levels of SeMet tested; this indicates that endothelial cells in the normal vasculature, which will not bind to the FP (since PS is not externalized), will not be affected by these concentrations of SeMet.

Cytotoxicity results for the two breast cancer cell lines are shown in Figures 5 and 6. For MCF-7 cells with FP present, there was significant killing at days 2 and 3 with 50 – 1000 μM SeMet (Figure 5, $p < 0.001$). Cell killing without FP present was not significant on day 3 until the SeMet concentration reached 1000 μM. MDA-MB-231 cells showed a greater sensitivity to the SeMet than MCF-7 and endothelial cells (Figure 6); significant cell cytotoxicity was observed with the FP present on days 1, 2, and 3 with 10 – 1000 μM SeMet ($p < 0.001$). Even without the addition of the SeMet, binding of the FP alone produced significant cell killing. With no FP present, cell killing did not occur until the SeMet level was 1000 μM and was relatively small and not statistically significant ($p < 0.001$).

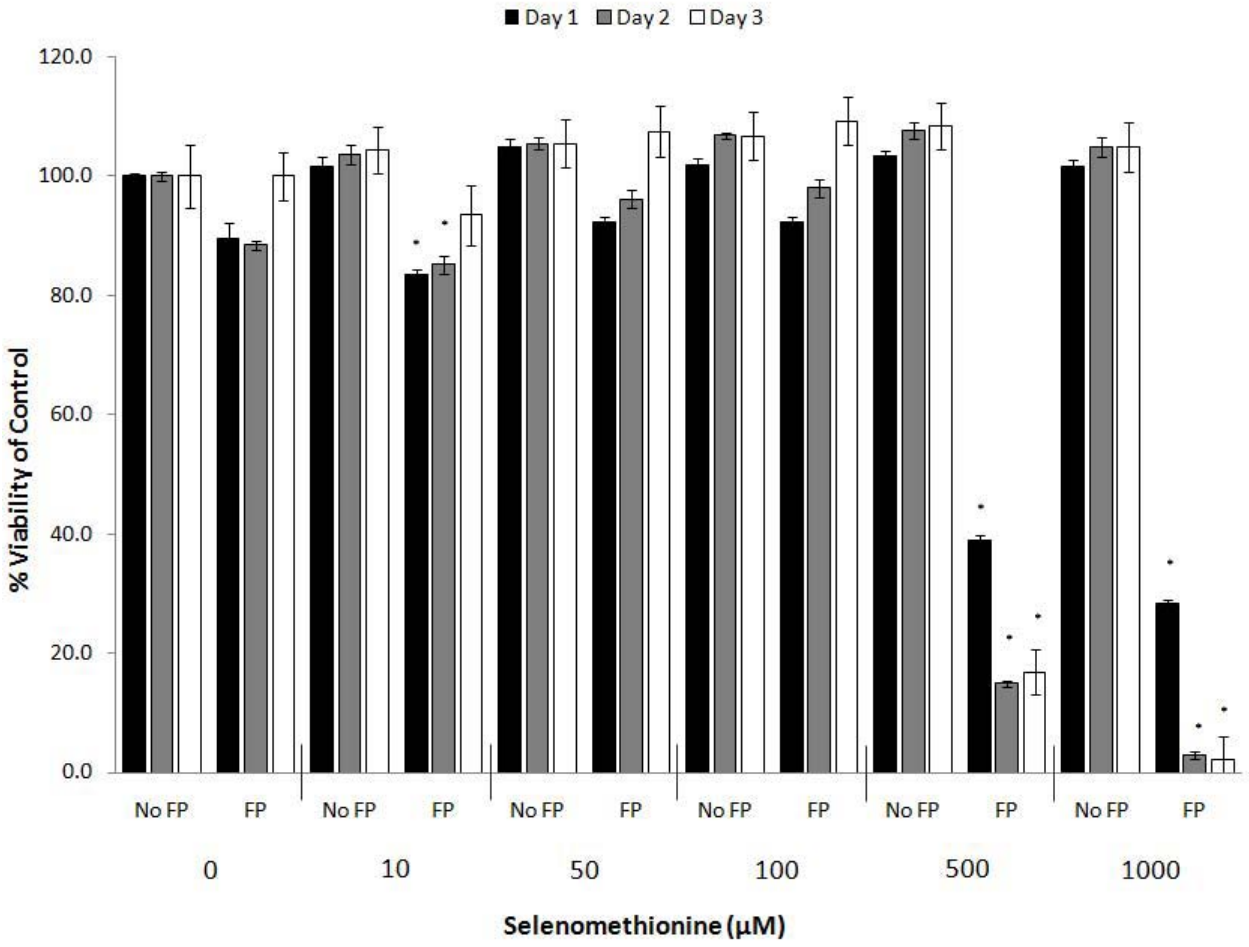

Figure 4: Effect of SeMet conversion to methylselenol on HAAE-1 endothelial cells. Cells were grown in medium adjusted to 1000 µM of L-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e. no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 µM SeMet) on the same day, and statistical significance was denoted by # ($p < 0.001$). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean ± SE (n = 3).

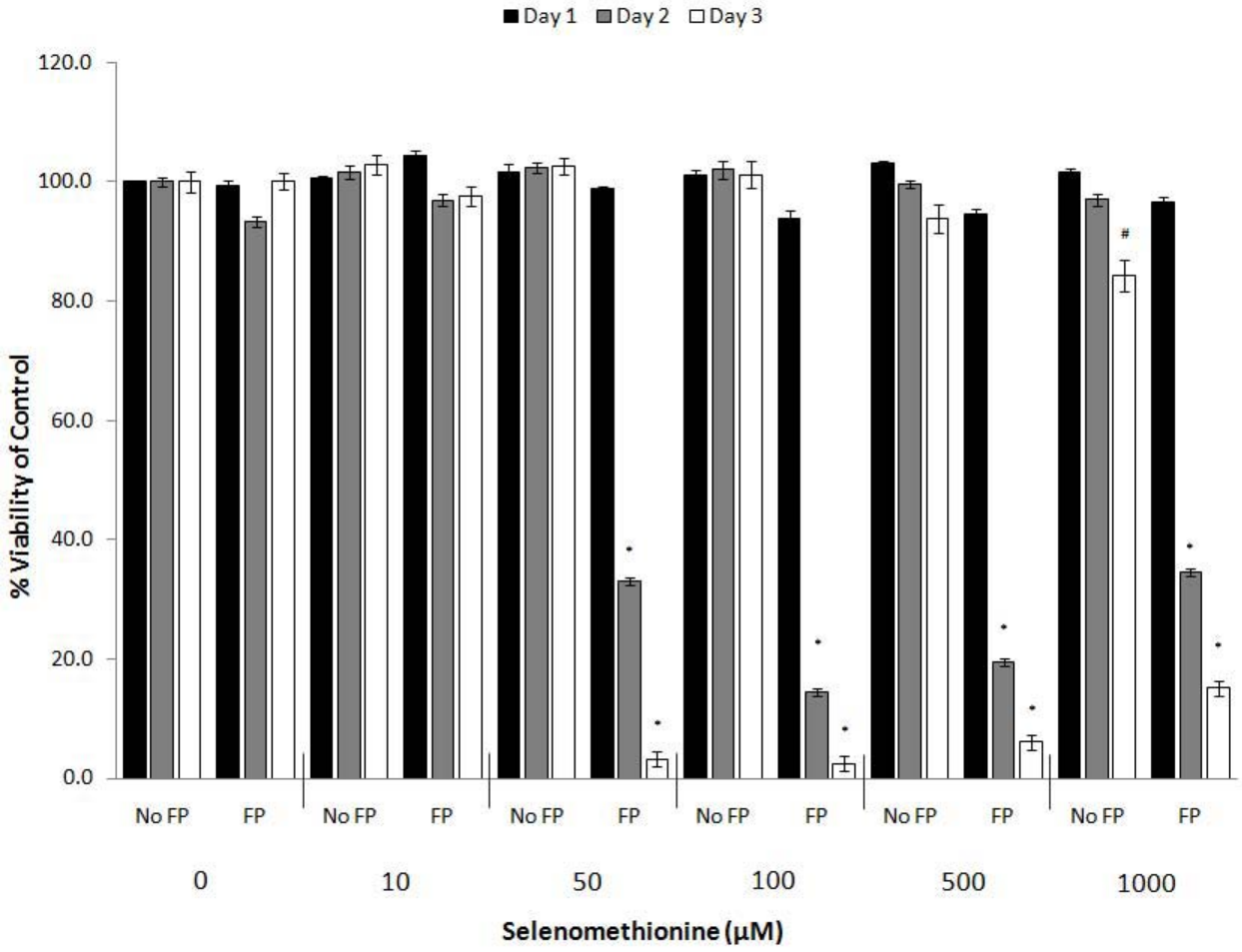

Figure 5: Effect of SeMet conversion to methylselenol on MCF-7 breast cancer cells. Cells were grown in medium adjusted to 1000 µM of L-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e. no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 µM SeMet) on the same day, and statistical significance was denoted by # ($p < 0.001$). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean ± SE (n = 3).

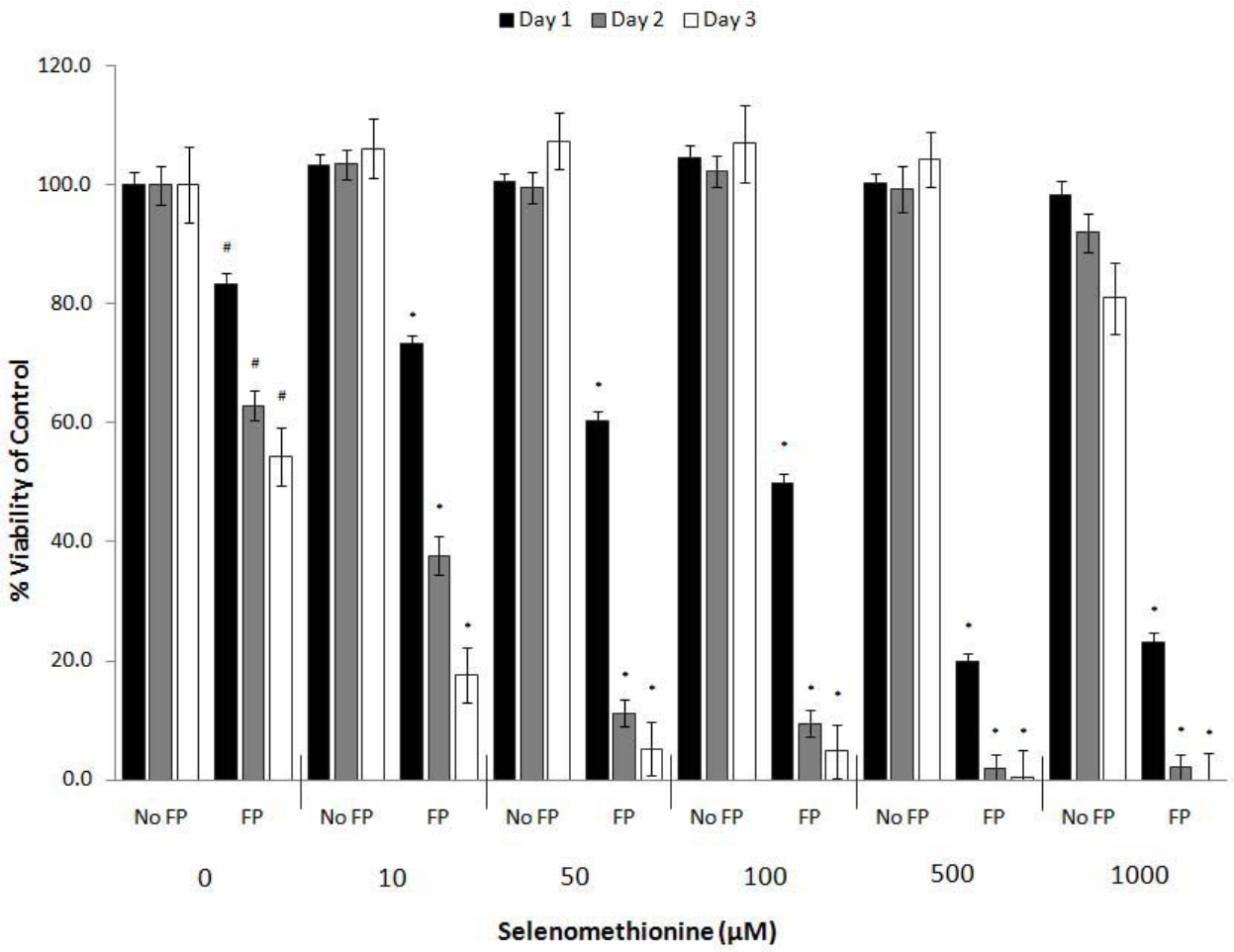

Figure 6: Effect of SeMet conversion to methylselenol on MDA-MB-231 breast cancer cells. Cells were grown in medium adjusted to 1000 µM of L-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e. no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 µM SeMet) on the same day, and statistical significance was denoted by # ($p < 0.001$). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean ± SE (n = 3).

Task 3 – Test of the anticancer activity *in vivo* of the L-methioninase-annexin V fusion protein in combination with selenomethionine prodrug on orthotopic breast tumor cell xenografts in nude mice.

The MDA-MB-231 breast cancer cell line transfected with green fluorescent protein (GFP) was obtained from Cell Biolabs, Inc. (San Diego, CA) and has been grown successfully *in vitro.* The growth rate of MDA-MB-231 cells transfected with GFP has been found to be about the same as MDA-MB-231 cells not transfected with GFP. We have obtained a GFP lentivirus from Cell Biolabs and plan to use it to transfect MCF-7 breast cancer cells within the next two months. We decided to transfect the cancer cells with GFP instead of β-galactosidase because it is generally superior in terms of sensitivity, speed, and non-invasiveness of the assay (Klein et al. 1997).

The time profile of the FP in the bloodstream after i.p. injection in nude mice was obtained (Figure 7). The FP was biotinylated using the same procedure as given in the year 1 annual report, and an ELISA method was used to measure the FP concentration (see the procedure in Appendix I). Four mice were used for each time point. For both of the levels of FP injected, the FP was at a peak level 1 hour after injection and then fell to an undetectable level by 8 hours from the injection. Based on these results, it is planned to inject 10 mg/kg for the enzyme prodrug tests in mice; as seen in Figure 7, a significantly higher level of FP was obtained for 10 mg/kg compared to 1 mg/kg (approximately 10 times higher), which indicates that the exposed PS on endothelial cells would be more likely to be saturated at an injection of 10 mg/kg compared to 1 mg/kg. We used an ELISA assay, rather than an assay for L-methioninase activity, to measure the FP concentration in the bloodstream, since our calculations indicated that the activity in the bloodstream would be too low to measure.

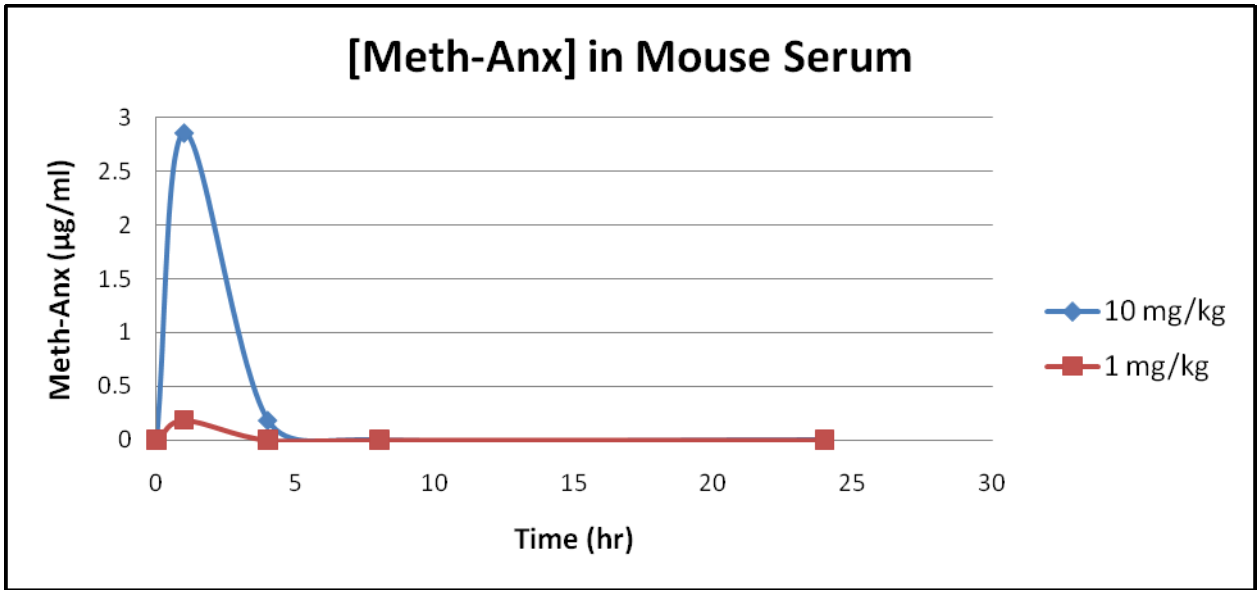

Figure 7. Time profile of L-methioninase-annexin V (Meth-Anx) fusion protein in the bloodstream of nude mice. Four animals were used for each time point.

The original plan for the tests of the enzyme prodrug in mice was to use injections of the SeMet prodrug at levels of 1, 10, 50, 100, and 200 mg/kg. We have consulted with Dr. C.V. Rao, a faculty member in the Hematology/Oncology Section at the University of Oklahoma Health Sciences Center, about these SeMet levels in mice because of his previous work in this area. He does not believe that we can inject SeMet at levels this high. Therefore, in order to potentially reduce the number of mice needed for the enzyme prodrug test, we did a toxicity test in which nude mice were injected at SeMet levels up to 60 mg/kg and then were monitored for 3 days (Table 1). Mice injected with SeMet levels up to 12 mg/kg all survived, while mice injected at two or five times this level had very low or zero survival rates. Based on these results, we are planning to use an injection level of SeMet of 10 mg/kg, which is the same as was used in a report in the literature of a methioinase cancer gene therapy in mice (Miki et al. 2001).

Table 1. Effect of SeMet dosage on survival in nude mice (6 per dosage level) for 3 days after injection.

SeMet dosage, mg/kg	Surviving Mice
0	6
6	6
12	6
24	1
60	0

The tests in nude mice of the quantification of PS exposure for MCF-7 and MDA-MB-231 breast cancer cells and of the effect of the enzyme prodrug with orthotopic tumor for MDA-MB-231 breast cancer cells are in progress at the time of writing this report, and results are not yet available.

KEY RESEARCH ACCOMPLISHMENTS

- Purified recombinant FP was produced in good purity (>97%) and relatively good yield (22 mg/liter of starting culture broth). Two mutations in the FP gene were corrected using site-directed mutagenesis.
- As indicated by measuring the dissociation constant (K_d), purified FP binds strongly to human endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells grown *in vitro*. Hydrogen peroxide was found to not be needed to induce exposure of PS on the endothelial cells. The following K_d values were determined: 0.5 nM for endothelial cells, 6.2 nM for MCF-7 cells, and 4.9 nM for MDA-MB-231 cells.
- The amount of FP bound on the three cell lines *in vitro* was found to decline steadily over 3 days, but there was still some FP bound at day 3.
- In tests of this enzyme/prodrug system *in vitro*, significant killing of the cells was found at a SeMet concentration of 500 μM for endothelial cells, 50 μM for MCF-7 breast cancer cells, and 10 μM for MDA-MB-231 breast cancer cells. With no FP present, significant cell killing was not observed for the endothelial cells and MDA-MB-231 cancer cells at up to 1000 μM SeMet and for MCF-7 cancer cells at up to 500 μM SeMet.
- A time profile of the FP in the bloodstream after i.p. injection in nude mice showed that the FP diminished to an undetectable level 8 hours after injection. The peak level of the FP was approximately 10 times greater for an injection of 10 mg/kg than for 1 mg/kg.
- A test of i.p. injection of SeMet in nude mice at various levels showed that up to 12 mg/kg could be used without toxic effects.

REPORTABLE OUTCOMES

Brent D. Van Rite, Yahya A. Lazrak, Magali L. Pagnon, Naveen R. Palwai, Peter S. McFetridge, and Roger G. Harrison, "Enzyme Prodrug Therapy Designed to Target L-Methioninase to the Tumor Vasculature," submitted for publication in *Cancer Letters.*

Brent D. Van Rite, Yahya A. Lazrak, Magali Pagnon, Vassilios I. Sikavitsas, Prithvi Bose, Carla Kurkjian, and Roger G. Harrison, "A Novel Enzyme/Prodrug for Targeting and Treatment of Solid Breast Tumors," poster presentation, Biomedical Engineering Society Annual Meeting, Austin, TX, October, 2010.

Brent D. Van Rite (speaker), Magali Pagnon, Yahya Lazrak, and Roger G. Harrison, "The Use of Targeted Enzyme/Prodrugs for the Treatment of Solid Tumors," oral presentation, 39th Annual Biochemical Engineering Symposium, Kansas State University, Manhattan, KS, April, 2010.

Harrison, R.G., "Enzyme Prodrug Cancer Therapy Selectively Targeted to Tumor Cells or Tumor Vasculature and Methods of Production Thereof," U.S. Patent Application, March, 10, 2010.

Yahya A. Lazrak, Brent D. Van Rite, Magali Pagnon, Arafat Tfayli, Peter S. McFetridge, and Roger G. Harrison (speaker), "Targeting the Vasculature of Solid Tumors with Enzyme/Prodrug

and Methionine-Depletion Therapy," oral presentation, American Institute of Chemical Engineers Annual Meeting, Nashville, November, 2009.

Harrison, R.G., Van Rite, B.D., Lazrak, Y.A., Pagnon, M., and McFetridge, P.S., "Targeting of Solid Tumor Vasculature with Enzyme/Prodrug and Methionine-Depletion Therapy," poster presentation, Biomedical Engineering Society Annual Meeting, Pittsburgh, October, 2009.

Harrison, R.G., "Enzyme Prodrug Cancer Therapy Selectively Targeted to Tumor Cells or Tumor Vasculature and Methods of Production Thereof," U.S. Provisional Patent Application, March, 2009.

Lazrak, Y.A. (speaker), Neves, L.F.F., McFetridge, P.S., and Harrison, R.G., "Novel Enzyme-Prodrug Therapy for Cancer," oral presentation, Biomedical Engineering Society Annual Meeting, St. Louis, October, 2008.

Harrison, R.G. (speaker), Lazrak, Y.A., Neves, L.F.F., McFetridge, P.S., and Tfayli, A., "Novel Enzyme-Prodrug Therapy for Cancer," oral presentation, American Institute of Chemical Engineers Annual Meeting, Philadelphia, November, 2008.

CONCLUSION

Recombinant FP was produced in good purity and yield from *E. coli*, using the FP gene with a completely correct sequence. The FP has been shown to bind strongly to PS exposed on human endothelial cells and MCF-7 and MDA-MB-231 breast cancer cells (dissociation constants ranging from 0.5 to 6.2 nM). The non-specific binding, obtained in the absence of Ca^{2+}, was subtracted from the total binding to obtain the specific binding. In a binding experiment over 3 days for each of the three cell lines, it was found that there was a steady decline in FP bound over this period, but there was still some FP bound at day 3.

Tests with the enzyme prodrug over a period of 3 days showed significant cell killing at 500 μM SeMet for endothelial cells, 50 μM SeMet for MCF-7 breast cancer cells, and 10 μM SeMet for MDA-MB-231 breast cancer cells. With no FP present, significant cell killing was not observed for the endothelial cells and MDA-MB-231 cancer cells at up to 1000 μM SeMet and for MCF-7 cancer cells at up to 500 μM SeMet. This data indicates that endothelial cells in the normal vasculature, which will not bind to the FP (since PS is not externalized), will not be affected by these concentrations (up to 1000 μM) of SeMet. At SeMet concentrations well below 500 μM, however, the cancer cells will be killed by toxic methylselenol being carried across the artery wall by fluid permeation.

Based on the time profiles of the FP in the bloodstream of nude mice, it is planned to inject 10 mg/kg for the enzyme prodrug tests in mice. The test of i.p. injection of SeMet in nude mice at various levels showed that up to 12 mg/kg could be used without toxic effects. For tests of the enzyme prodrug in nude mice (currently in progress), the FP and SeMet will both be injected at a level of 10 mg/kg. An FP level of 10 mg/kg will be used to inject mice in the test of the quantification of PS exposure for MCF-7 and MDA-MB-231 breast cancer cells (test currently in progress).

The results to date provide strong support for one of the basic ideas for the project, which is that the conversion of the SeMet to methylselenol at the surface of the tumor vasculature will lead to damage of the tumor vasculature; this damage will lead to clotting of the tumor vasculature, thus cutting off the oxygen supply of the cancer cells. In addition, the breast cancer cells will be killed by toxic methylselenol being carried across the artery wall by fluid permeation. The effect of methylselenol on normal cells outside of the tumor is expected to be minimal or none because it will be greatly diluted by the bloodstream before it reaches the normal cells. Also, SeMet in the bloodstream will not be converted to methylselenol in the normal vasculature, because PS will not be exposed, and the SeMet will be held at a level that will not damage the normal vasculature. Therefore, side effects should be minimal.

REFERENCES

Klein D, Indraccolo S, von Rombs K, Amadori A, Salmons B, Gunzburg WH. 1997. Rapid identification of viable retrovirus-transduced cells using green fluorescent protein as a marker, Gene Ther 4:1256-60.

Miki, K, Xu, M, Gupta, A, Tan YB, Al-Refaie, W, Bouvet, M, Makuuchi, M, Moossa, AR, Hoffman, RM. 2001. Methioninase cancer gene therapy with selenomethionine as suicide prodrug substrate, Cancer Res 61:6805-10.

APPENDIX I

ELISA Protocol to Measure Methioninase-Annexin V FP in Nu/J Mouse Serum

Purpose: To determine the time required for biotinylated methioninase-annexin V clearance from the bloodstream.

In Vitro Plate Preparation

1. Use streptavidin-coated 96 well plates from Thermo Fisher Scientific.

2. Add 50 µl of each serum sample to wells (2 dosage levels – 10 mg/kg and 1 mg/kg; 4 time points per dosage level – 1 hr, 4 hr, 8 hr, and 24 hr post-injection; 4 samples with no injection as the blank).

3. Cover the plate with adhesive cover and incubate for 60 min at 37^{o}C.

4. Shake out the plates into a sink. Wash the plates with *Wash Buffer* 4 times by adding 200 µl and shaking out the *Wash buffer* into a sink. Pat plates dry by inverting on paper towel.

5. Add 50 µl of annexin V polyclonal antibody (rabbit) diluted to 1.25 µg/ml (recommended by Abcam) in *Diluting Buffer* to each well.

6. Cover the plate with adhesive cover and incubate for 60 min at 37^{o}C.

7. Shake out the plates into a sink. Wash the plates with *Wash Buffer* 4 times by adding 200 µl and shaking out the *Wash Buffer* into a sink. Pat plates dry by inverting on paper towel.

8. Add 50 µl of anti-rabbit IgG-HRP conjugate (secondary antibody) diluted to 1:1,000 (initially at about 1 mg/ml) using *Diluting Buffer* to each well.

9. Cover the plate with adhesive cover and incubate for 60 min at 37^{o}C.

10. Shake out the plates into a sink. Wash the plates with *Wash buffer* 4 times by adding 200 µl and shaking out the *Wash buffer* into a sink. Pat plates dry by inverting on paper towel.

11. Add 50 µl of OPD (chromogenic substrate) solution to each well.

12. Cover the plate with adhesive cover and incubate for 30 minutes at room temperature in the dark.

13. Read absorbance at 450 nm.

Reagent List:

1. Diluting Buffer
   - 0.5 g Tween 20

- 2.5 g BSA (0.25%)
- Add PBS to 1 L.

NOTE: Do not add sodium azide with the HRP-IgG method.

2. Wash Buffer
   - 5 g Tween 20 (0.05%)
   - Add PBS to 1 L.
3. O-phenylenediamine (OPD) Solution
   - 1 phosphate-citrate capsule
   - Add DI H_2O to 100 ml
   - Weigh out OPD powder at 0.4 mg/ml and put in centrifuge tube wrapped in aluminum foil
   - Immediately before use, add 40 µl of 30% H_2O_2 to initiate the reaction
   - Add necessary amount of buffer solution to tube and use as soon as possible